# Cell Damage due to Hydrodynamic Stress in Fluorescence Activated Cell Sorters

**A Bachelors of Science Thesis**

**Prepared in Accordance to Requirements for:**

**Graduation with Distinction in Chemical and Biomolecular Engineering**

**At The Ohio State University**

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**The Ohio State University**

**2009**

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**Abstract**

A common technique in biological and medical research involves sorting cells using a fluorescence activated cell sorter (FACS). These sorters can achieve a great degree of purity, close to 98% [1], which is useful if the only necessity is the sorting itself. In many cases, the cells must be used for further analysis after being sorted; however, researches have noticed a decrease in cell viability and slowed growth after sorting. It is hypothesized that this damage is due to the hydrodynamic stress that the FACS imposes upon cells. This study will focus on the FACS Aria, while furthering the study of THP-1 and beginning analysis of a new cell line, which belongs to the same family as THP-1, the U-937 cell line. Experiments with the cell sorter will be conducted in order to gain an understanding of the cell damage and the channel in the Aria will be modeled in order to later simulate the fluid flow through the Aria. Additionally, a micro-fluidic device will be utilized in order to study the U-937 cell line's particular response to hydrodynamic stress. This research hopes to yield a more in depth understanding into the stress that cells undergo during cell sorting, how cells respond to this stress, such as growth kinetics, cell cycle changes, and amount of cell damage, and the variability in cell sensitivity to hydrodynamic stress.

**Acknowledgements**

First, I would like to thank Claudia Berdugo, without whom this entire project would not have been possible. Secondly, I want to thank Dr. Jeffrey Chalmers for giving me the opportunity to do undergraduate research.

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## 1. Introduction

A fluorescence activated cell sorter (FACS) is often used in medical and biological research to differentiate between cells for a variety of characteristics, such as protein expression. Cells can easily be stained and passed through a cell sorter, which quickly determines the presence of a specific protein or whichever cell function being targeted. However, a major problem that has developed involves the fact that after cells are sorted they are difficult to grow and the cell viability decreases. It was hypothesized that the main deleterious effect on the cells involved the hydrodynamic stress that is applied to cells, during the cell sorting process. Additionally, it has been seen that the type of cell being sorted affects the degree of damage because each cell line has a different level of sensitivity to hydrodynamic stress. Chinese hamster ovary, CHO, and THP-1, a human leukemia line, cells have been previously analyzed, but this research will further the studies on THP-1 as well as begin studying a new cell line, U-937, from the family of monocytic leukemia cells to which THP-1 belongs. Overall, this study aims to better understand fluorescence activated cell sorters, particularly the FACS Aria, the cellular response to the hydrodynamic stress associated with cell sorters, and the different sensitivities of the cell lines, such as THP-1 and U-937.

### 1.1 Background on FACS

A fluorescence activated cell sorter applies a significant amount of stress, due to the requirements of cell sorting. Cells flowing through a cell sorter are subjected to stress due to a rapid contraction, which occurs through a series of components, that force the cells into a single file. While in this line, the cells are exposed to a laser(s), and based on their absorbance, the cells are charged and deflected into different compartments. The diagram below portrays a general schematic of the laser exposure and cell deflection that occurs.

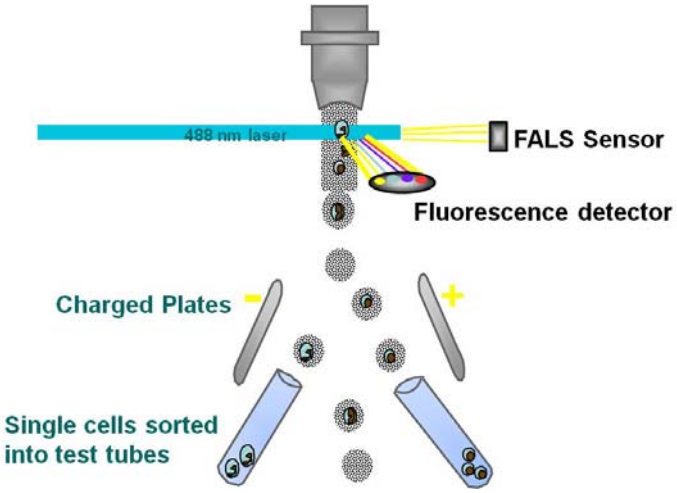


**Figure 1: Schematic of FACS**

The hydrodynamic forces that cell sorters apply to cells cause cell death by necrosis and apoptosis. Necrosis is commonly referred to as passive cell death or "cell murder"; however, apoptosis, or active cell death, involves the cell actively taking a role in its death or in other words the cell commits suicide. Necrosis generally involves a toxin or outside source, such as hydrodynamic force, breaking the membrane and causing death. Apoptosis occurs from different signals, which could involve environmental issues such as pH or lack of nutrients or, as shown in previous research, can involve mechanical stress [2]. The external signal does not cause an actual break in the membrane, but it triggers the cell to undertake a series of steps that ultimately lead to its death.

In previous research, the FACS Vantage was investigated, and in the case of this sorter, the contraction and interrogation point, where the cells are exposed to the laser(s), took place solely in the nozzle. However, the FACS Aria has several components, which are hypothesized to inflict stress. The most significant parts of the Aria include the nozzle and the cuvette, as they have the greatest degree of contraction. The interrogation point occurs in the cuvette, and the nozzle is used to force the flow into droplets so that they are more easily deflected. The manner in which these pieces fit together is interesting because no smooth transition exists between

components. A more detailed description will be provided as a part of the results and discussion of the modeling that will be done.

1.2 Assessing Hydrodynamic Stress

Different methods, stress tensor, the amount of rotation, and energy dissipation rate, have been proposed to express the amount of stress inflicted on cells. The stress tensor accounts for both shear and extensional components of the flow, and the amount of rotation considers the idea that cells do in fact rotate, meaning the amount of strain on a particular part of the cell membrane is not constant [1]. For this study, the energy dissipation rate (EDR) was used to indicate the amount of hydrodynamic stress applied to cells. The EDR is the amount of work done on a fluid [1],[2]. Another definition is that EDR measures the amount of irreversible internal energy increase per volume [3]. It is beneficial to use EDR to assess the amount of stress because it is a scalar quantity that encompasses both shear and extensional components of the stress and is derived out of a fundamental understanding of fluid dynamics. The cell culture media flowing through the nozzle is assumed to have the same properties as water, which is an incompressible Newtonian fluid. According to this assumption the calculation for EDR can be seen below: [1],[2]

**Equation 1: EDR Calculation**

$$\varepsilon = \mu \sum_i \sum_j (\nabla U + \nabla U^T)_{ij} \nabla U_{ji}$$

where $\mu$ is viscosity, $\nabla U$ is the velocity gradient tensor, and $\nabla U^T$ is the transpose of $\nabla U$.

Due to the complicated nature of this equation and the geometries of different cell sorters, computational fluid dynamics software (CFD) can be utilized to determine the EDR value. The flow associated with cell sorters generally lies in the laminar flow region. Therefore, the CFD calculations are in fact accurate because the flow does not enter the turbulent region [1].

1.3 Torture Chamber

Another device used to analyze hydrodynamic stress is a micro-fluidic convergent and divergent device commonly referred to as a "torture chamber." The rapid contraction and expansion in the channel models the geometry in a cell sorter. The torture chamber has already been extensively modeled and simulated; therefore, the amount of EDR at various velocities through the chamber is known. The diagram below shows a sketch, including dimensions, of the torture chamber.

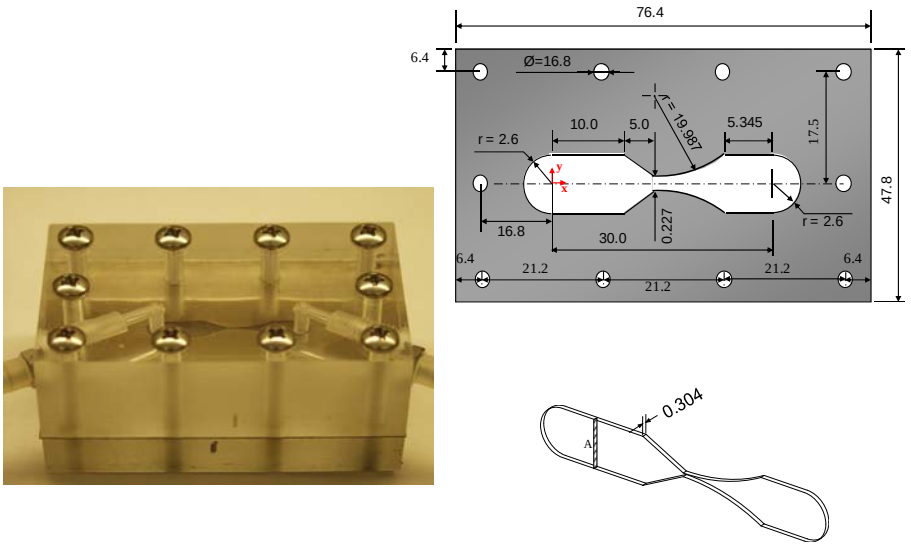


**Figure 2: Sketch of Torture Chamber [1]**

Experiments with the torture chamber involve single passes through the channel at varied velocities followed by damage assessment. Since the torture chamber has already been extensively modeled, each velocity applies a known degree of stress; therefore, a damage assessment after the cells pass through the channel can be related back to the amount of stress applied, providing information on cell sensitivity. Previous experiments with the torture chamber demonstrate that different cell lines have varied degrees of sensitivity to stress, Figure 3. The graph shows the percentage of damage to the cell sample as it exposed to different levels of EDR in the torture chamber. As seen in the figure, CHO cells have proven to be very robust;

however, the monocytic leukemia cell line THP-1 was observed to be far more sensitive to hydrodynamic stress.

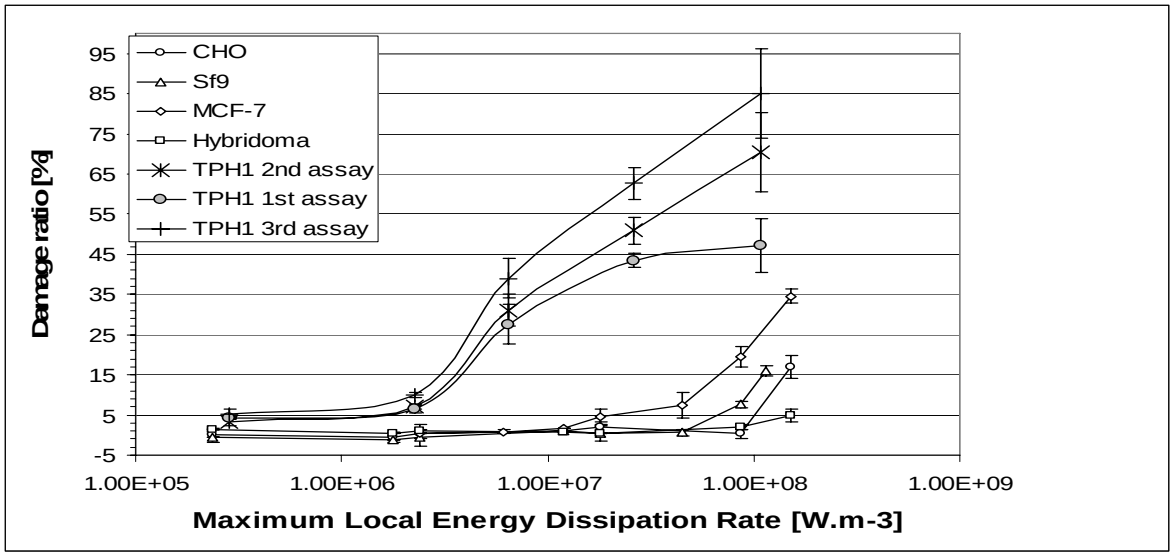


**Figure 3: Cell Sensitivity to Varied EDR [1]**

It is interesting to note that not all human cells are as sensitive as THP-1 cells. The MCF-7 cell line, which is a human breast cancer cell line, displays a sensitivity behavior much more similar to the CHO cells than THP-1. Therefore, it is important to investigate whether THP-1 is a special case of increased sensitivity or if other cell lines, such as ones from the same family of leukemia cells, possess the same level of sensitivity. The U-937 line belongs to the same family as THP-1. This cell line must be analyzed and compared to the results of the THP-1 studies in order to gain a deeper understanding of the sensitivity of this family of cells. The U-937 cell line will be analyzed similarly to previous experiments, utilizing the fact that the torture chamber has already been modeled and the EDR that it inflicts upon cells is known at varied velocities.

1.4 Damage Analysis

Different methods exist to determine the amount of cell damage that occurs. One common method involves a staining technique in which the stain enters through a break in the membrane. The die, trypan blue, is allowed to enter the cells and using a hemacytometer under a microscope to hand-count the live cells, those without the color, and the dead cells. This methodology is utilized for growth kinetics analysis. The growth kinetics is studied to determine if hydrodynamic stress affects the cell growth after sorting. The general cycle for cellular growth involves the lag, exponential growth, stationary, and death phases. The figure below shows a typical growth curve. Researchers using the cell sorters have noticed that cells do not grow as quickly; therefore, studying growth kinetics will allow us to understand what is different about the cell cycle in these sorted cells. Specifically, this study will look at the duration of the lag phase as a potential reason for this slowed growth.

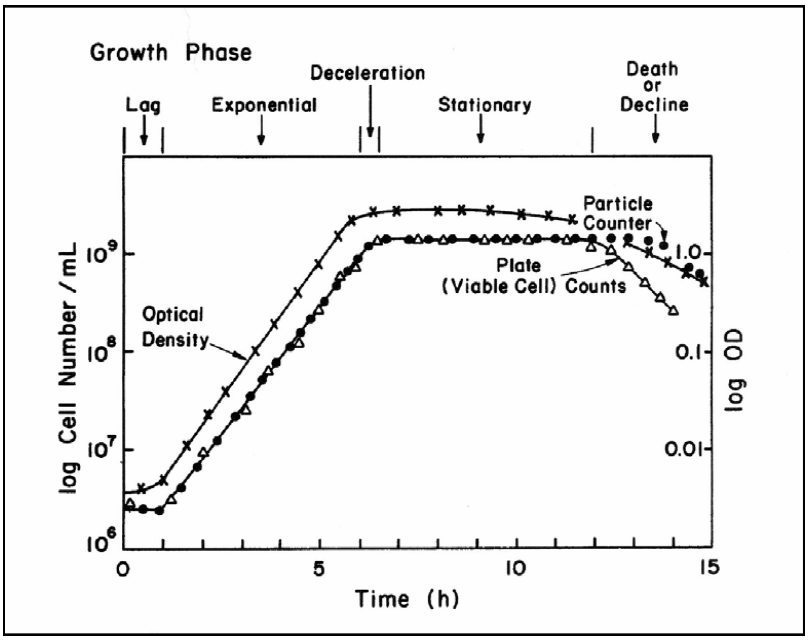


**Figure 4: General Description of Cellular Growth**

An example of growth being affected after exposure to stress involves a study, which investigated the growth of human Aortic Smooth Muscle Cells (hASMC) after exposure to shear

stress. After a 24 hour growth period, the cells were exposed to known amounts of shear stress in a parallel plate flow chamber. The cells were allowed to grow for 24 hours and then compared to a control that was seeded at the same time but not exposed to the stress. The study compared the number of cells and concluded that the growth rate was significantly less in the cells exposed to the stress [4]. The previous study did not do an extensive analysis into the actual kinetics; however, the current research investigates the specifics of the growth kinetics in order to determine potential differences between the control and the sorted cells.

Another analysis of growth kinetics involves the changes in glucose and lactate concentrations over the period of growth. As the cells grow, they digest the glucose as an energy source and produce lactate. In order to demonstrate this trend, the supernatant from the cell sample is removed and tested for glucose and lactate concentrations, after the cells have been counted. The expected result, here, involves a consumption of the glucose and production of lactate as the cells grow.

Another method for assessing cell damage involves measuring the amount of lactate dehydrogenase (LDH), which is a cytosolic enzyme released as cells die, present in the supernatant. A reagent is utilized to convert a tetrazolium salt (INT) into formazan red, and the amount of color can be measured and directly related to the number of lysed cells, through the construction of a standard curve with known concentrations of cells in solution. The reactions occurring during this assay can be found below [5].

**Equation 2: Convert NAD+ to NADH**

$$NAD^{+} + lactate \rightarrow pyruvate + NADH$$

**Equation 3: Convert Tetrazolium Salt to Formazan Red**

$$NADH + INT \rightarrow NAD^{+} + formazan(red)$$

The previously discussed study on the growth of hAMSC cells performed an LDH analysis to remove cell damage and death due to the stress itself as a possible cause for the fewer amount of cells present in the culture [4]. Although the study differs from the research presented here, it is important to note that similar studies have been performed in order to determine how cells respond to hydrodynamic stress.

Another hypothesis being tested in this study involves the cell's life cycle. Previous research has observed that those cells in the Gap 2 (G2) phase are more susceptible to damage. This phase occurs after DNA synthesis, and further cell growth occurs before moving into mitosis, where the cell actually divides. Significant protein synthesis occurs during this phase in preparation for cell division. Specifically, the cell is very large at this point, filled with double the necessary components in preparation for cell division. Additionally, the cell membrane begins to pinch in during this phase. Due to this increased strain on the membrane during this phase, an increase in sensitivity to the hydrodynamic stress occurs. The diagram below shows the cell cycle in more detail, providing descriptions for each phase.

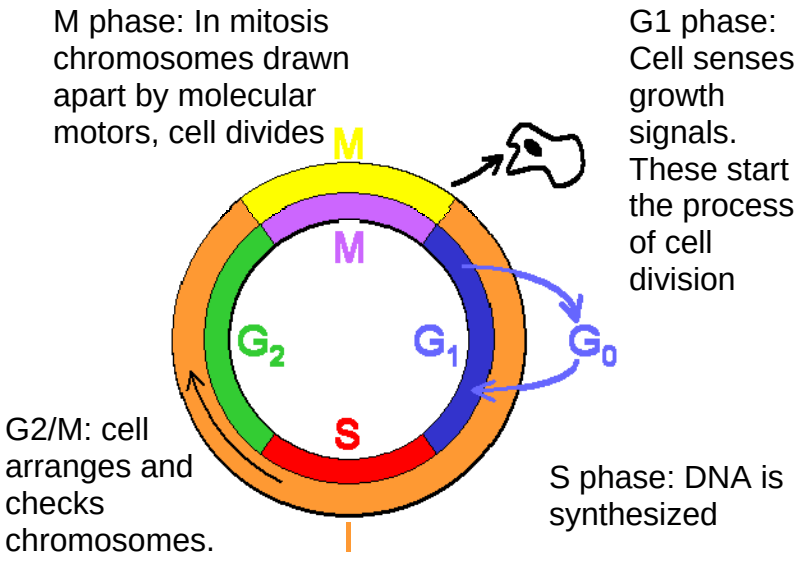


**Figure 5: Cell Cycle Description**

Using a flow cytometer, this study will evaluate the idea that more cell damage occurs to those cells in the G2 phase while undergoing hydrodynamic stress. Below is an example of the phase profile that the flow cytometer provides. The first red peak reflects the G1 phase and the second, smaller peak portrays the G2 phase.

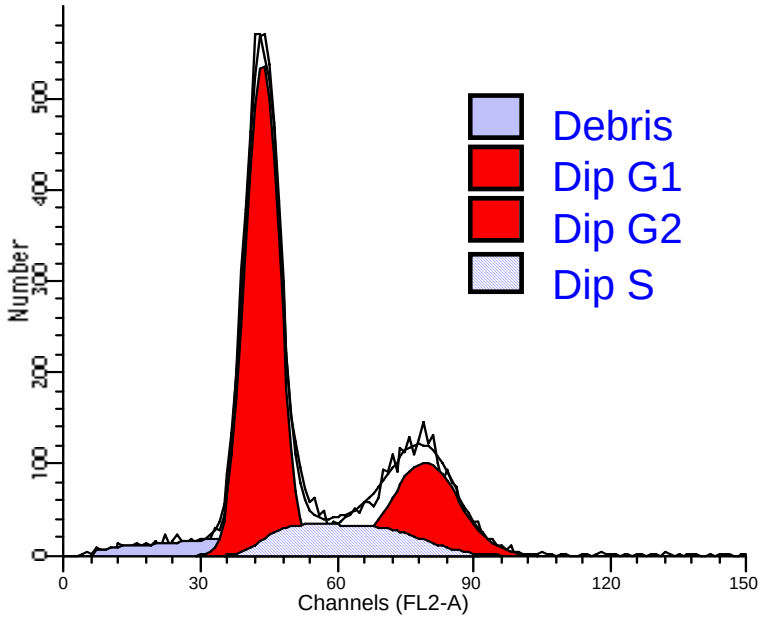


**Figure 6: Phase Profile from Calibur**

1.5 Media Effects

A couple different media effects were studied over the course of this project. One involved the potential protective ability of fetal bovine serum (FBS), and the second tested a different media option for seeding cells after the sorting.

The addition of FBS serum to the cell media before sorting cells may work to protect cells from extensive damage. One study on the effects of turbulent shear stress in a bioreactor analyzed the protective ability of FBS by exposing the cells to this shear stress with and without 10% FBS. The paper also discussed possible mechanisms for this protection: purely physical in that it reduces shear forces or biological, such as causing a modification to the cell membrane. It was observed that the addition of FBS in fact reduced the intensity of turbulence in the bioreactor

[4]. Additionally, previous research in an unrelated area saw that FBS could be used to decrease the occurrence of zona hardening of mouse oocytes during cryonization. FBS stops the interaction between the cortical granules and zone pellucida, thereby preventing zona hardening [5].

Additionally, the type of media used for cell cultures after sorting was studied in order to determine a new methodology that could improve growth. A conditional media with 50% fresh media and 50% old media in which the cells were previously growing was compared to growth in fresh media. Sorted cells were used to seed the media in order to compare the growth kinetics. The hypothesis here involves the fact that as cells grow they produce growth factors that help the surrounding cells grow. Therefore, the conditional media would contain these growth factors and enable the cells to grow better after being sorted.

1.6 Cell Cultures Utilized

The two cell lines, THP-1 and U-937, used for this study were both part of the monocytic leukemia cell family. THP-1 cells have been extensively used in previous studies; however, the U-937 cell line is new to this form of study. The THP-1 cell line is responsible for acute monocytic leukemia and U-937 is defined by ATCC® as the cause of histiocytic lymphoma and abcam® as a human leukemic monocyte lymphoma The U-937 cell line was first established by Dr. K. Nilsson's laboratory: Sundstrom and Nilsson (Int. J. Cancer 17: 565-577, 1976). These cell lines can be obtained from ATCC®: The Global Bioresource Center, which provides information on growth, doubling time, etc. It is important to note that both of the cell lines have a biosafety level of 1, meaning they are relatively harmless and are not known to cause disease in healthy adults. Both cell lines are small and the picture of THP-1 cell culture provides a scale on

the specific size of these cells. As one can see, the cells are much smaller than the nozzle sizes: 70 and 85 micron diameters.

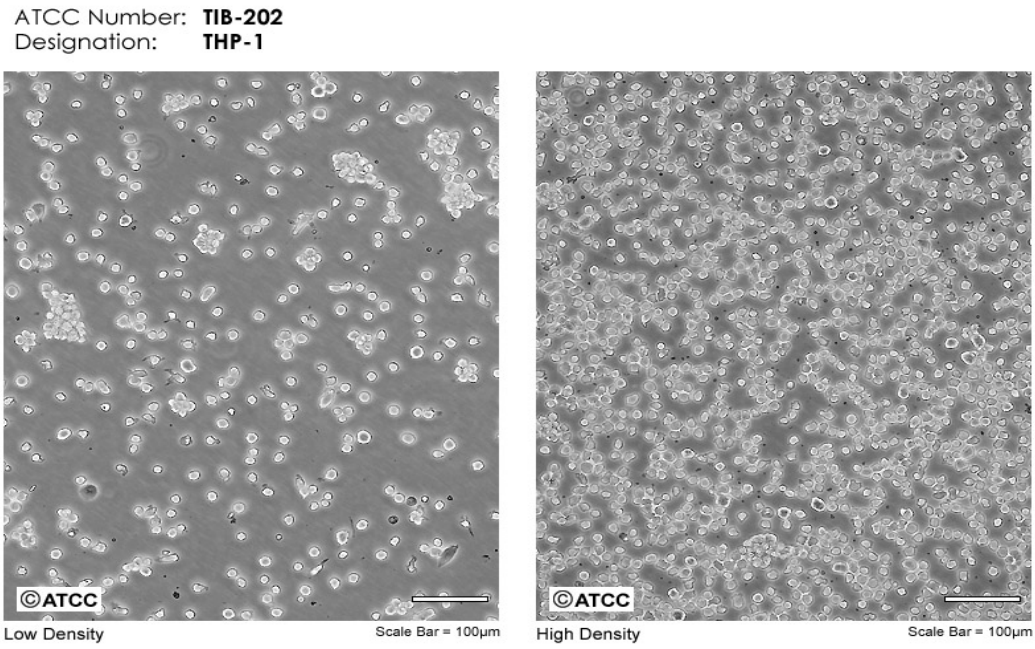


**Figure 7: THP-1 Cell Line from ATCC**

## 2. Methodology

The study uses several different methodologies to not only apply hydrodynamic stress to cells, but also analyze the amount of damage that occurs. The following sections will describe the procedures used to apply stress as well as the analysis of said stress.

### 2.1 Torture Chamber Experiments

To analyze the response of the U-937 cell line to hydrodynamic stress, this study performed three single-pass experiments in the torture chamber. The experimental setup involves a small container to which the cell culture is added, a 60 mL syringe, mechanical pump, the torture chamber, and plastic tubing to connect the system. Using the mechanical pump, the syringe is filled to approximately 30 mL, and the pump then pushes on the syringe forcing the fluid through the torture chamber. The volumetric velocity of the flow is altered via a computer, which is connected to the pump. The different speeds include 10, 30, 50, 70, and 90 mL/min. Two passes are made for each velocity, and the ten total passes are properly randomized. Typically, the 90 mL/min velocity is repeated a third time because of the difficulty of collecting sample at this velocity, and if error is suspected from any other pass, it can be repeated as long as enough cell culture remains.

The process of collecting samples involves purging the system before and after the pass through the torture chamber. It is important to purge the system of the cells remaining from the previous pass, which is typically at a different velocity, so that the data accurately reflects the damage that occurs at the specified velocity. The following procedure is used for sampling each pass: purge 5 mL, take 3 mL before the culture enters the torture chamber as control, allow the culture to flow through the torture chamber and purge 5 mL, and finally take 7 mL for the post

torture chamber sample. The samples, both the before and after, are analyzed for cell damage using the lactate dehydrogenase test, which is described in detail, below.

2.2 Cell Sorting in the FACS Aria

Experiments with the FACS Aria can be done to understand the actual cell damage that occurs during sorting and compare the damage to what is expected based on the EDR calculation that will be done in a future study. This project performed four sorts, including three with THP-1 cells and one with U-937.

The first sorting experiment involved the THP-1 cell line with the 85 micron diameter nozzle and approximate cell concentration of 5x10^6 cells/mL. Three different sorts, each specifically designed for a particular cell damage analysis, were performed with the same batch of cells. One sort was geared towards growth kinetics analysis. This sort suspended the cells in 1 mL of RPMI with 10% FBS. A control of 1 mL with the same cell concentration was allowed to sit open to the air during the duration of the sort. After sorting, the collected cells were used to seed two different medias: an RPMI media with 10% FBS and a conditional media comprised of 50% fresh RPMI and 50% media that the cells were previously growing in. The cells were seeded into T-flasks with concentrations of 0.1x10^6 cells/mL in a total of 16 mL. Control flasks were seeded with the RPMI and 10% FBS media at the same cell concentration. Another sort was performed using the same media of RPMI and 10% FBS; however, this sort focused on cell cycle analysis. A control was taken for the before sorting and with the cells collected after sorting, a cell cycle analysis was performed as described in detail, later. A third sort, using media with 0% FBS, focused on LDH analysis. Some of the cells from the first sort with the 10% FBS media were also utilized for the LDH analysis portion of this sorting experiment in order to compare these results. It is expected that the cells in 0% FBS media will have more

damage than those cells in the 10% FBS. The same procedure as above was utilized, but instead of seeding flasks for growth kinetics, the cells were used for LDH analysis as described in detail, later in this paper.

The second sorting experiment used THP-1 cells with the 70 micron nozzle diameter in the Aria. In this case, only two sorts were performed due to a lower amount of cells. The first sort utilized a concentration of 8.65x10^6 cells/mL, and the cells from this sort were used for the growth kinetics and cell cycle analysis. As described before, the cells were suspended in RPMI and 10% FBS media and then sorted, while a control was left open to the air during the duration of the sort. The sorted cells were used to seed T-flasks with RPMI and 10% FBS, but no conditional media flasks were seeded for this experiment. Some remaining cells were used to perform the cell cycle analysis. A second sort was done for the LDH analysis by suspending a concentration of 5.63x10^6 cells/mL in RPMI with 0% FBS. Some of the sample from the sort in 10% FBS media was again used for LDH analysis to compare the two media.

The third sorting experiment involved the new cell line U-937. It was performed in a similar manner as the second sorting experiment; however, the two sorts utilized cell concentrations of approximately 4x10^6 cells/mL.

A fourth sort was conducted using a similar procedure as the first sort except the 70 micron nozzle was utilized for this sort. Again, the growth kinetics analysis involved looking at differences among the control and the sorted cells in both conditional and fresh media. The LDH and cell cycle analysis procedures were the same as before.

2.3 Damage Analysis

As previously discussed, lactate dehydrogenase is released upon cell lysis. Therefore, measuring the amount of LDH in the supernatant provides an understanding of the amount of

cell death that occurs. The overall procedure involves the construction of a standard curve to which the actual samples can then be compared and measuring the samples, themselves.

Constructing the standard curve involves killing known concentrations of cells by freezing them for two hours. This allows for a direct comparison of the amount of LDH measured to the amount of dead cells. A sample is taken from the cells being utilized for the experiment in order to make a stock solution of 0.5*10^6 cells/mL to seed the 12 samples at varying dilutions of 1 mL in each sample. In order to ensure accuracy of the measurements two sets of these 12 are made. These 24 samples are then placed in -80 F for two hours, during which the known number of cells will die and release LDH. Fifty microliters of sample are added to a well-plate followed by the addition of a reagent which reacts with the LDH and converts tetrazolium salt into formazan red. The reagent is allowed to react for 30 minutes at which point a stop solution is added and the plate can be analyzed for LDH based on the level of color present. With increasing concentration of dead cells, the amount of LDH increases.

Those cells that have been exposed to hydrodynamic stress are treated in a similar manner. The supernatant is removed and added to a plate well. In cases where a high amount of cell damage is suspected, a dilution of the sample is necessary so that the reading remains in the realm of the standard curve. The samples are treated with the reagent and stop solution and analyzed for LDH in the same manner as the standard curve. The amount of LDH present in each sample can then be compared to the standard curve constructed in order to determine the amount of cells that were killed during the exposure to stress.

Another method for determining the amount of damage that occurs bases the calculation on the amount of LDH present if 100% of the cells were killed during the cell sorting process. For this evaluation, a sample was removed from the solution of cells before sorting and frozen to

kill these cells. The equation below was used to determine the damage ratio. The equation uses the absorbance for the experimental sample, control, 100% death, and media to determine the percent damage.

**Equation 4: Percent Damage from LDH Assay**

$$\frac{Experiment\ Abs - Control\ Abs}{100\%\ Abs - Media\ Abs} \times 100 = \%\ \text{Damage}$$

As previously described, the sorting experiments involve the seeding of several T-flasks for growth kinetics analysis. The flasks are kept in an incubator to insure proper growth. For the first week after sorting or until the cells have reached the stationary phase of the growth curve, the cells are sampled and counted twice a day. It is important to properly resuspend the cells in the media, and approximately 200$\mu$L are removed from each T-flask. For the first few samples, only a 1:2 dilution of trypan blue and the cell sample is made for counting. As described above the trypan blue enters only damaged cells. The cells are counted with a hemacytometer. Once the cells enter into the exponential growth phase, the cells should be diluted to 1:4 using trypan blue and PBS.

In addition to counting the cell sample with the hemacytometer, the supernatant is removed to test for the glucose and lactate concentrations, using the YSI Biochemical Analyzer. In some cases, dilutions must be made because the YSI can only measures glucose up to 2.5 g/L and lactate to 0.5 g/L. It is important to vortex the sample before allowing the YSI to measure in order to ensure the concentration is homogenous. After vortexing, the sample simply needs to be presented to the aspirating needle of the YSI and a reading is taken.

Additionally, this study will analyze potential changes in the cells' life cycles. This analysis involves taking a control sample before sorting and fixing the cells to remain in the

particular life cycle phase, S, G1, and G2, that they are currently. In order to fix the cells, first suspend the sample in 300 $\mu$L of cold PBS. While vortexing, slowly, drop by drop, add 700 $\mu$L of ethanol, and leave this in ice (~-20°C) for one hour. Centrifuge the sample and wash 2-3 times with PBS. Remove the supernatant and then resuspend in 500 $\mu$L of the staining agent, propidium iodide (PI). Once the cells are suspended in the PI solution, they must remain at room temperature for thirty minutes and then analyzed. A sample of the sorted cells is then procured and treated in the same manner. These samples can then be tested by a flow cytometer called the Calibur. The Calibur monitors the amount of cells in each phase by evaluating the amount of DNA present, and it provides graphical and tabular forms of data so that the pre-sorting control can be directly compared to the sorted cells and analyzed for any differences.

2.4 Modeling the Aria

While flowing through the flow cytometer the cells undergo a certain amount of stress particular to the sorter being used. The FACS Aria is constructed in a particular manner that differs from other sorters, such as the previously studied FACS Vantage. To better understand how the structure affects the amount of stress applied to the cells, the flow cytometer is modeled and simulated based on conditions typical of cell sorting. Gambit is the modeling program associated with the commercial CFD program, FLUENT®; therefore, Gambit will be utilized to create the mesh so that it can be simulated in FLUENT®. The components to be modeled include the nozzle, including both the 70 and 85 micron diameters, and the cuvette. The simulation will be conducted in a future study, but this study will establish the fluid dynamics and geometry of the Aria.

## 3. Results

The following sections relay the results for the different experiments, damage analyses, and modeling work that was performed throughout the course of this study.

### 3.1 Single Passes with Torture Chamber

For each of the three single pass experiments, LDH analysis was conducted. The damage ratio for each pass at a specific velocity was determined, and these velocities were correlated to their respective EDR's. All of the data for each set of experiments was averaged to obtain a set of data relating the percentage of cell damage to the EDR. This data was then graphed along with data from previous studies with different cell lines, such as THP-1 and CHO.

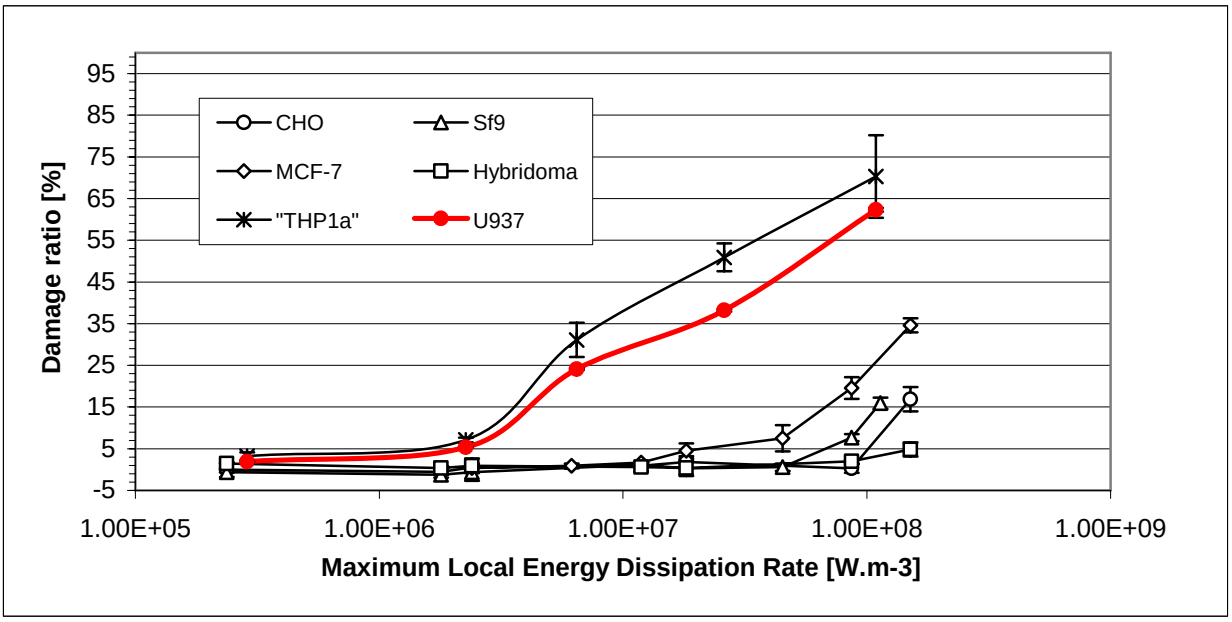


**Figure 8: Cell Damage versus EDR (U-937)**

### 3.2 LDH Analysis for Sorting Experiments

From the LDH analysis for each sort, the damage percentage was tabulated, and the results for the 85 and 70 micron nozzle sorts with THP-1 are included below.

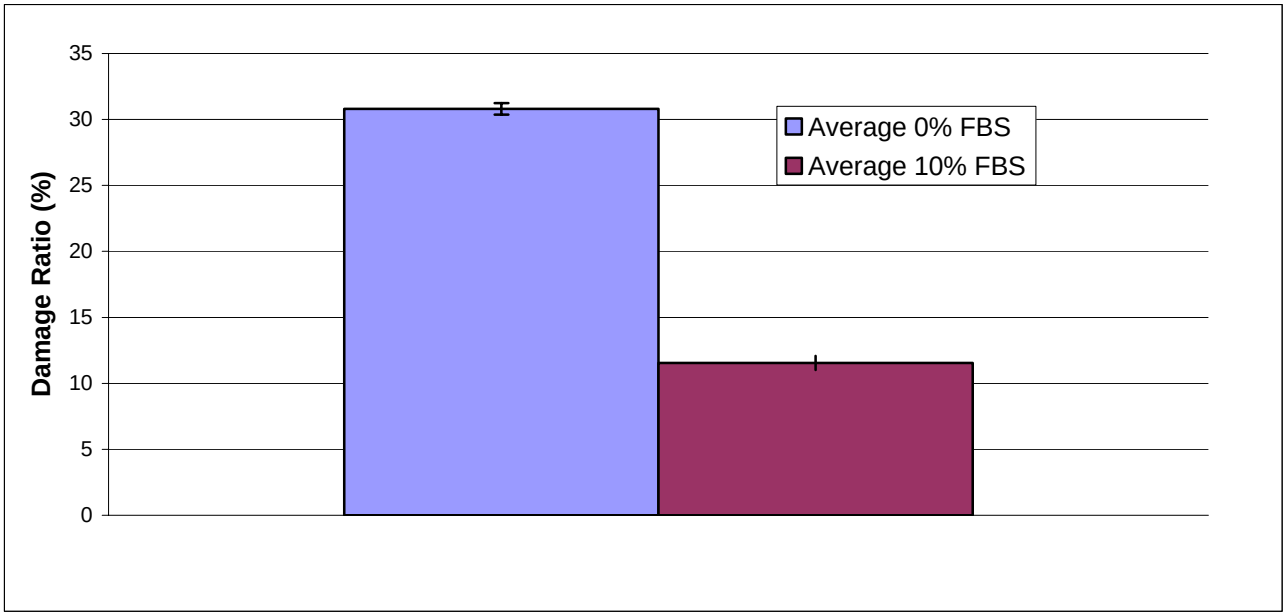


**Figure 9: Cell Damage after Sorting with 85 micron Nozzle**

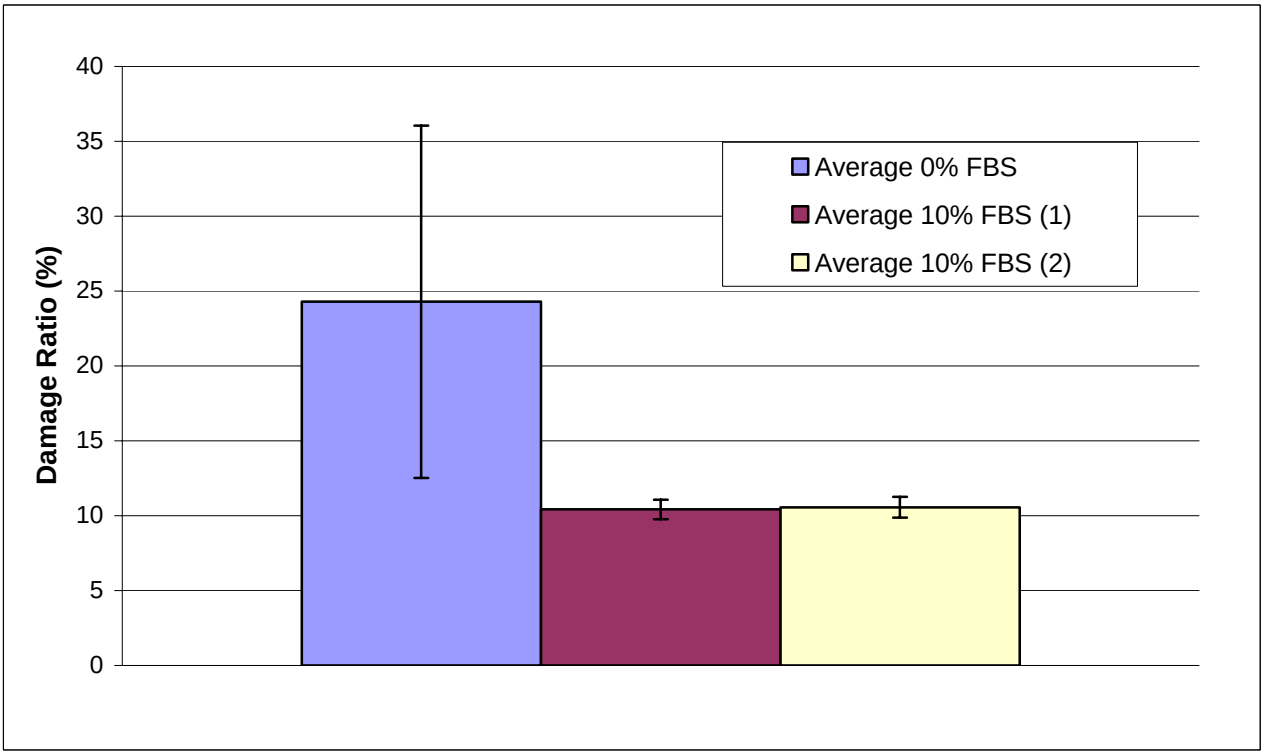


**Figure 10: Cell Damage after Sorting with 70 micron Nozzle**

3.3: Growth Kinetics

From the four different sorting experiments, the growth kinetics was analyzed. The first sorting involved control, sorted cells in 10% FBS, and sorted cells in conditional media. The figure below expresses the results of the growth kinetics for this experiment.

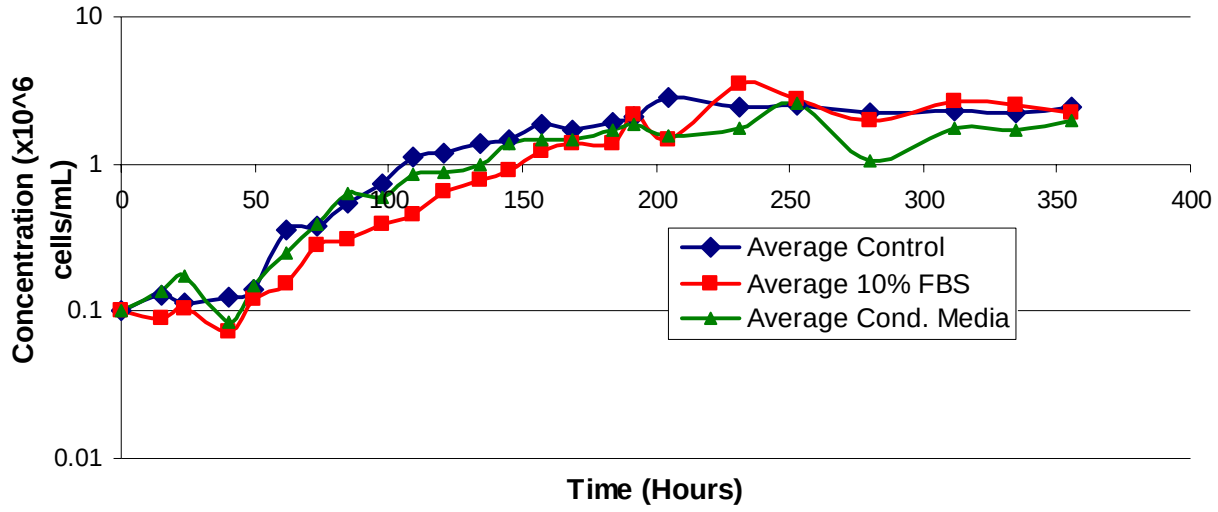


**Figure 11: Growth Kinetics after Sorting with 85 micron Nozzle**

In order to demonstrate that the sorted cells grow differently from the control, one must focus on the lag and exponential growth phases. Figure 12 shows this particular area of interest for the growth kinetics.

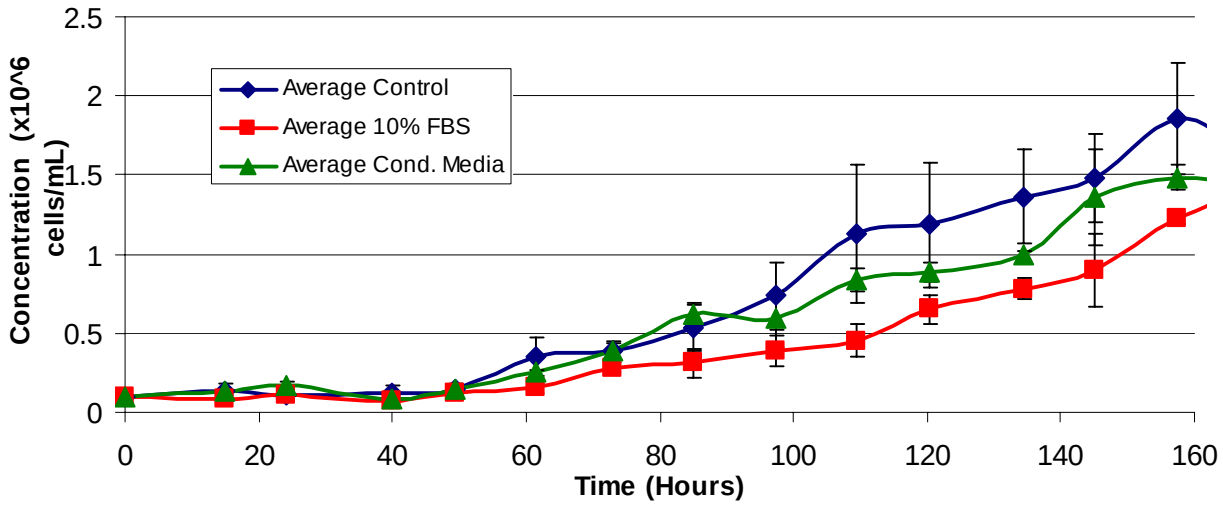


**Figure 12: Growth Kinetics (85 micron) Highlighting Growth**

The growth rates for the control, conditional media, and fresh media were determined to be 0.0181, 0.0186, and 0.0189 hr^-1, respectively.

Additionally, the glucose and lactate concentrations were analyzed to complement the cell counting, and the results from this analysis for the first sort are found in Figures 13 and 14.

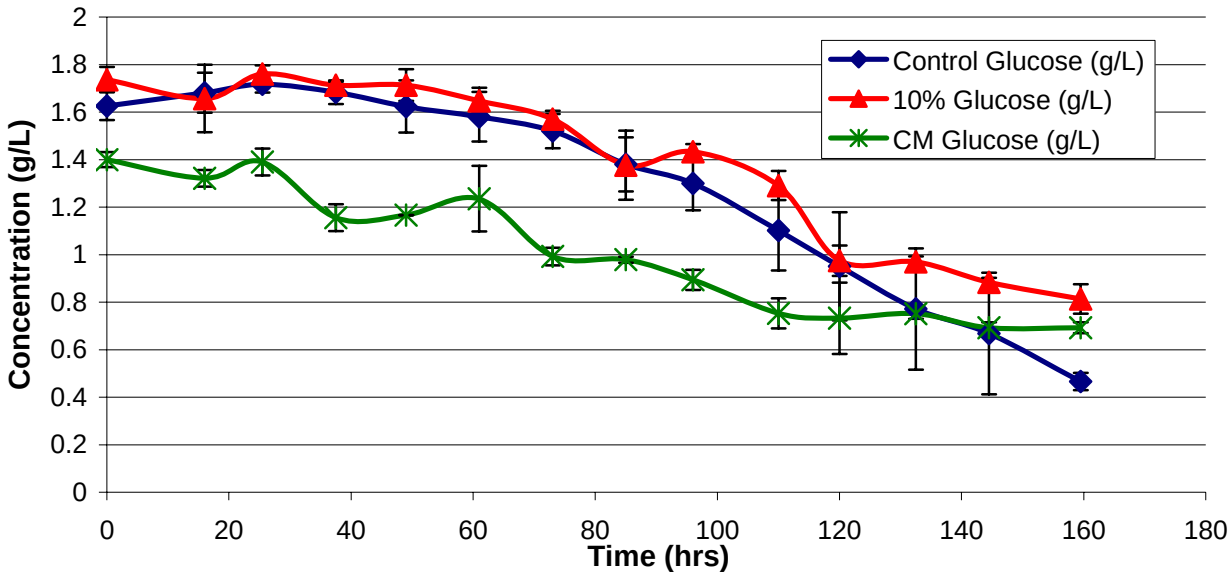


**Figure 13: Glucose Concentrations (85 micron+THP-1)**

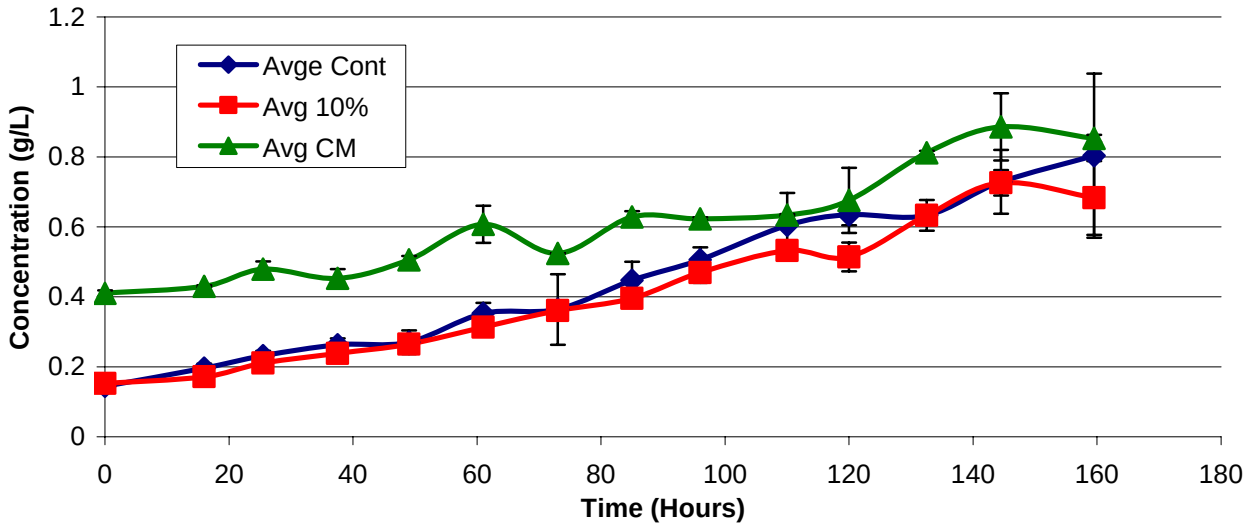


**Figure 14: Lactate Concnetrations (85 micron+THP-1)**

The fourth sort conducted involved the THP-1 cells and the 70 micron nozzle. The growth kinetics from this sort is included here, and these reflect similar results as the first sort.

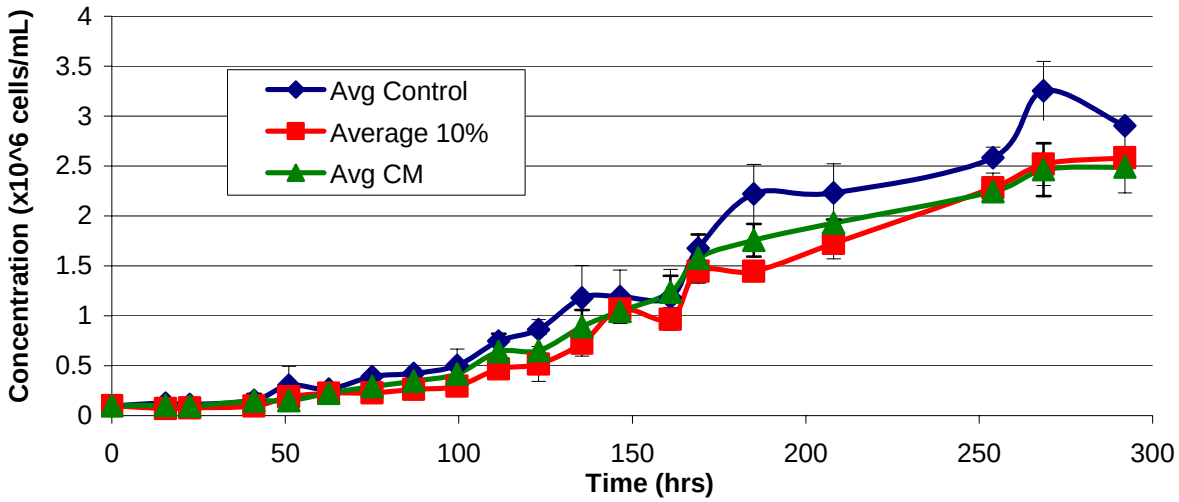


**Figure 15: Growth Kinetics after Sorting in 70 micron Nozzle**

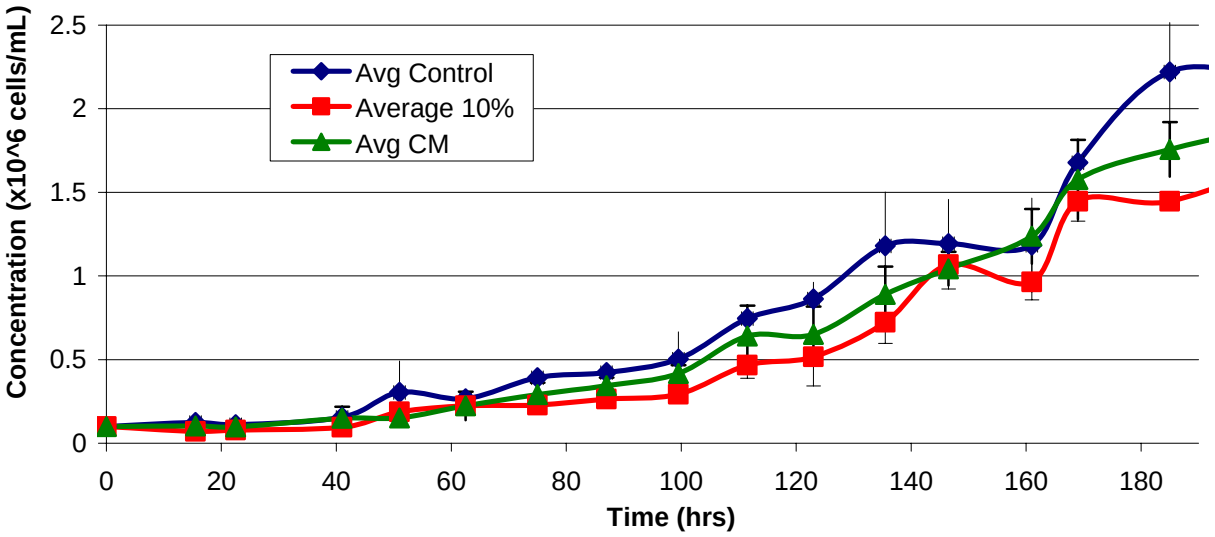


**Figure 16: Growth Kinetics (70 micron) Highlighting Growth**

The growth rates for the control, conditional media, and fresh media were determined to be 0.024, 0.0214, and 0.0254 hr^-1, respectively.

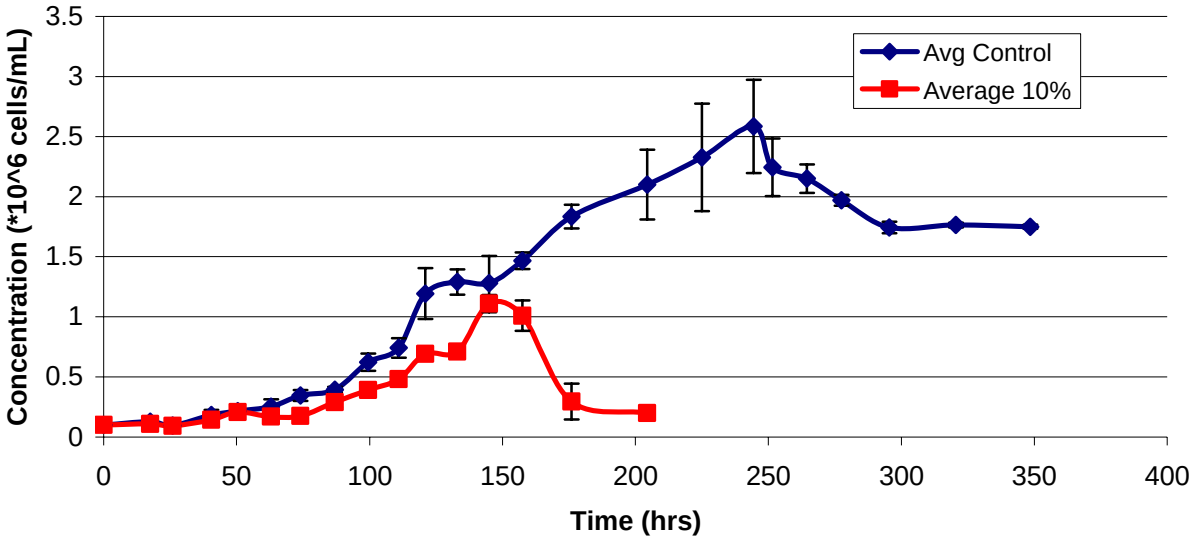


**Figure 17: Growth Kinetics of U-937 after Sorting 70 micron Nozzle**

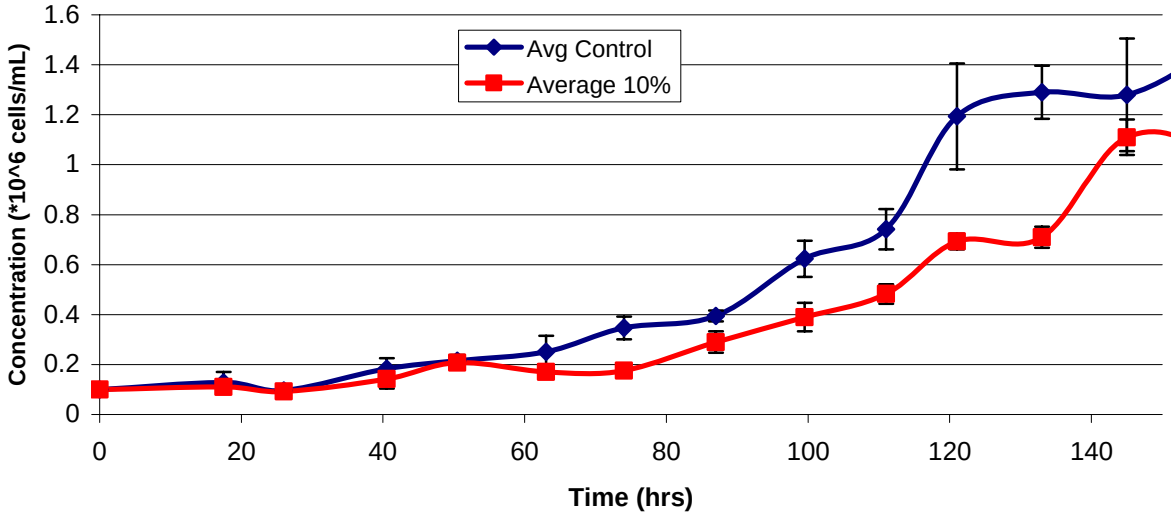


**Figure 18: Growth Kinetics of U-937 (70 micron) Highlighting Growth**

Again the growth rates for the control and the fresh media were determined to be 0.0203 and 0.023 hr^-1, respectively.

3.4 Cell Cycle Analysis

In order to test the hypothesis about increased cell damage to those cells in the G2 phase, the Calibur was utilized to obtain the following graphs and histograms. The tables provide specific information regarding the amount of cells in each phase in the percent gated column.

M4 represents the G2 phase, and the other significant phase, G1, is represented by M2. The results included here are the sorts with THP-1 using the 85 and 70 micron diameter nozzles. Some more results can be found in the appendix.

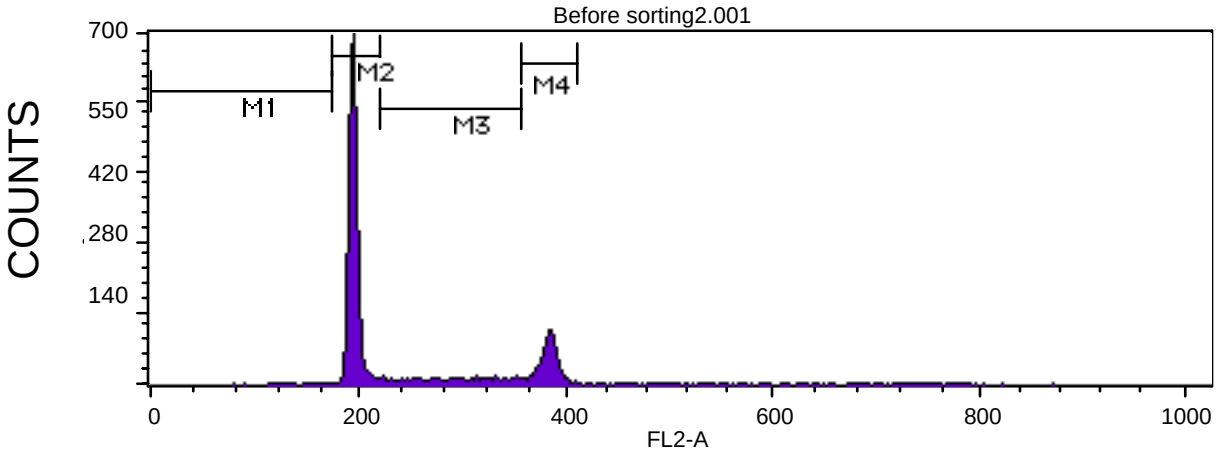


**Figure 19: Cell Cycle Analysis before Sorting (85 micron)**

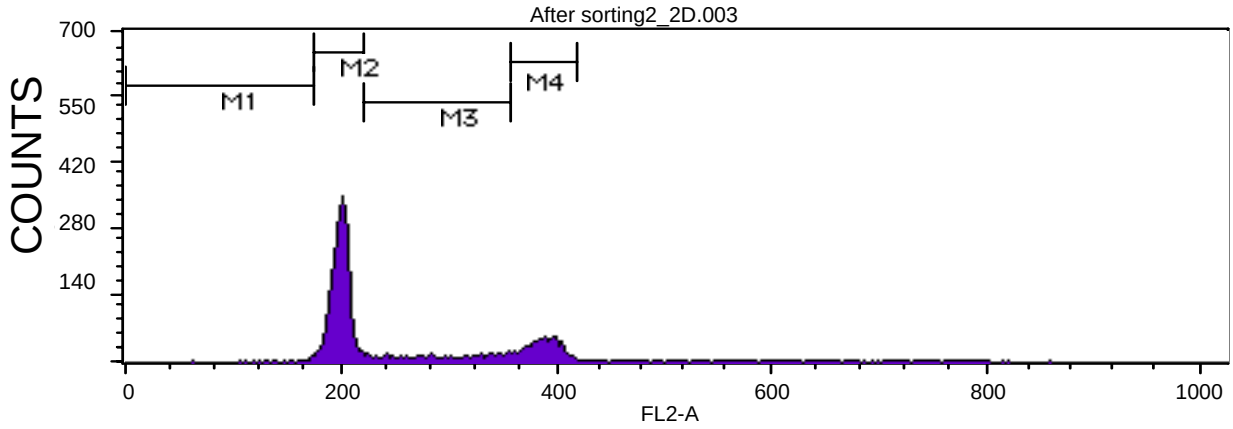


**Figure 20: Cell Cycle Analysis after Sorting (85 micron)**

**Table 1: Cell Cycle Analysis Data before Sorting (85 micron)**

File: Before sorting2.001 Sample ID: Before sorting2
Acquisition Date: 11-Apr-09 Gate: G3

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | CV | Median | Peak Ch |
|---|---|---|---|---|---|---|---|---|---|
| All | 0, 1023 | 9211 | 100.00 | 92.11 | 258.59 | 243.64 | 39.53 | 198.00 | 195 |
| M1 | 0, 175 | 41 | 0.45 | 0.41 | 142.12 | 140.29 | 15.46 | 146.00 | 123 |
| M2 | 175, 222 | 5830 | 63.29 | 58.30 | 196.03 | 195.96 | 2.61 | 195.00 | 195 |
| M3 | 222, 358 | 1274 | 13.83 | 12.74 | 292.51 | 289.63 | 13.93 | 293.00 | 224 |
| M4 | 358, 420 | 1811 | 19.66 | 18.11 | 382.43 | 382.30 | 2.53 | 383.00 | 383 |

**Table 2: Cell Cycle Analysis Data after Sorting (85 micron)**

File: After sorting2_2D.003 Sample ID: After sorting2_2D
Acquisition Date: 11-Apr-09 Gate: G3

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | CV | Median | Peak Ch |
|---|---|---|---|---|---|---|---|---|---|
| All | 0, 1023 | 9187 | 100.00 | 91.87 | 264.54 | 248.84 | 39.87 | 205.00 | 201 |
| M1 | 0, 175 | 77 | 0.84 | 0.77 | 158.71 | 156.81 | 13.81 | 170.00 | 174 |
| M2 | 175, 222 | 5609 | 61.05 | 56.09 | 198.60 | 198.44 | 3.93 | 199.00 | 201 |
| M3 | 222, 358 | 1418 | 15.43 | 14.18 | 294.11 | 291.06 | 14.25 | 296.00 | 356 |
| M4 | 358, 420 | 1770 | 19.27 | 17.70 | 386.49 | 386.23 | 3.63 | 387.00 | 387 |

The following results come from the second sorted, which used the 70 micron diameter nozzle and sorted THP-1 cells.

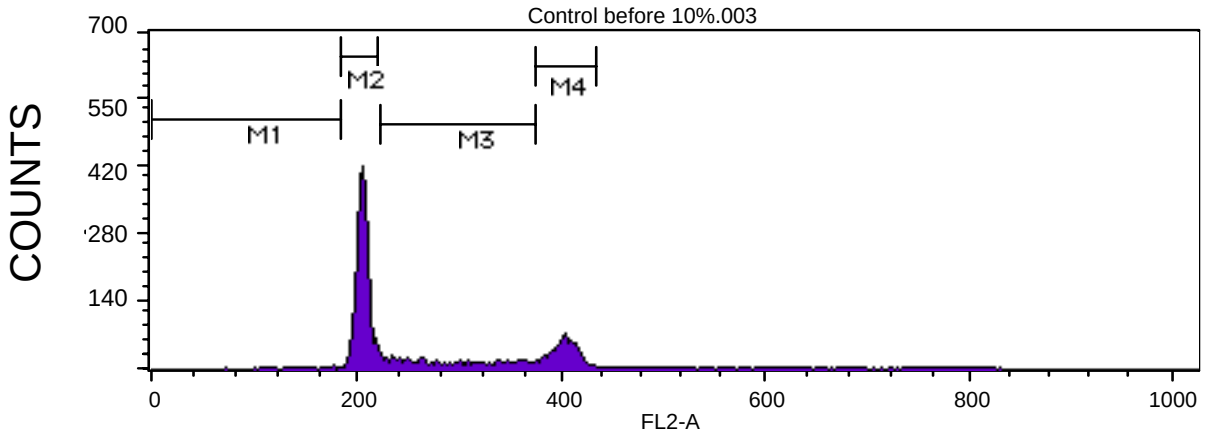


**Figure 21: Cell Cycle Analysis before Sorting (70 micron)**

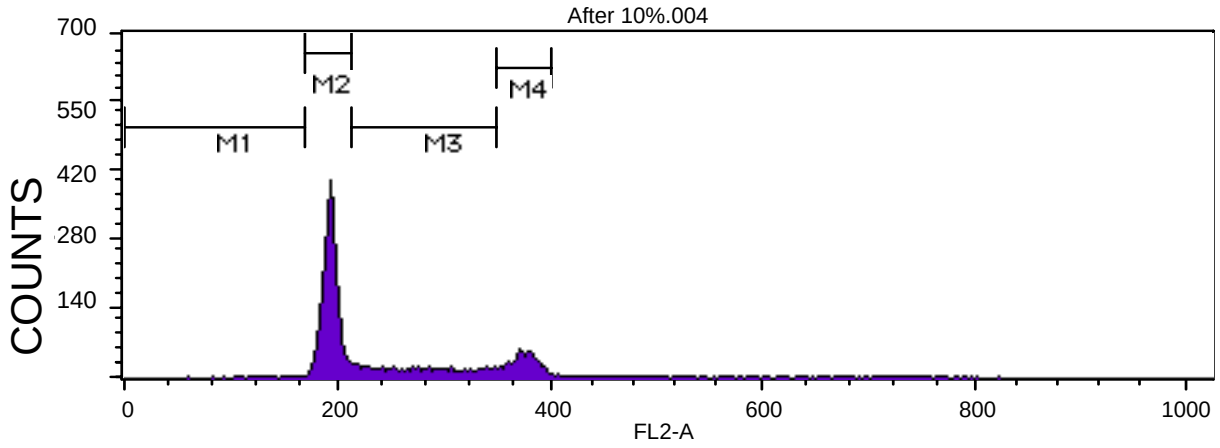


**Figure 22: Cell Cycle Analysis after Sorting (70 micron)**

**Table 3: Cell Cycle Analysis Data before Sorting (70 micron)**

File: Control before 10%.003 Sample ID: Control before 10%
Acquisition Date: 21-Apr-09 Gate: G3

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | CV | Median | Peak Ch |
|---|---|---|---|---|---|---|---|---|---|
| All | 0, 1023 | 9167 | 100.00 | 91.67 | 278.61 | 261.98 | 39.88 | 213.00 | 206 |
| M1 | 0, 186 | 90 | 0.98 | 0.90 | 150.99 | 148.54 | 17.35 | 153.00 | 178 |
| M2 | 186, 222 | 5043 | 55.01 | 50.43 | 206.18 | 206.10 | 2.76 | 206.00 | 206 |
| M3 | 224, 376 | 1879 | 20.50 | 18.79 | 294.12 | 290.28 | 16.18 | 291.00 | 224 |
| M4 | 376, 435 | 1755 | 19.14 | 17.55 | 402.40 | 402.22 | 2.96 | 403.00 | 404 |

**Table 4: Cell Cycle Analysis Data after Sorting (70 micron)**

File: After 10%.004 Sample ID: After 10%
Acquisition Date: 21-Apr-09 Gate: G3

| Marker | Left, Right | Events | % Gated | % Total | Mean | Geo Mean | CV | Median | Peak Ch |
|---|---|---|---|---|---|---|---|---|---|
| All | 0, 1023 | 9260 | 100.00 | 92.60 | 251.68 | 238.68 | 37.01 | 199.00 | 193 |
| M1 | 0, 170 | 66 | 0.71 | 0.66 | 137.14 | 135.04 | 16.43 | 135.50 | 132 |
| M2 | 170, 214 | 5423 | 58.56 | 54.23 | 193.00 | 192.87 | 3.70 | 193.00 | 193 |
| M3 | 214, 350 | 2043 | 22.06 | 20.43 | 277.95 | 274.94 | 14.72 | 276.00 | 218 |
| M4 | 350, 402 | 1445 | 15.60 | 14.45 | 374.08 | 373.89 | 3.14 | 374.00 | 371 |

3.5 Modeling Work

The simulation work on the FACSAria began with modeling the flow channel in Gambit. Figure 23 shows the modeled channel with a nozzle diameter of 85 microns, and due to the complicated nature of the connection, Figure 24 has been added to show this connection between the cuvette and the nozzle, both the rapid contraction and expansion pieces.

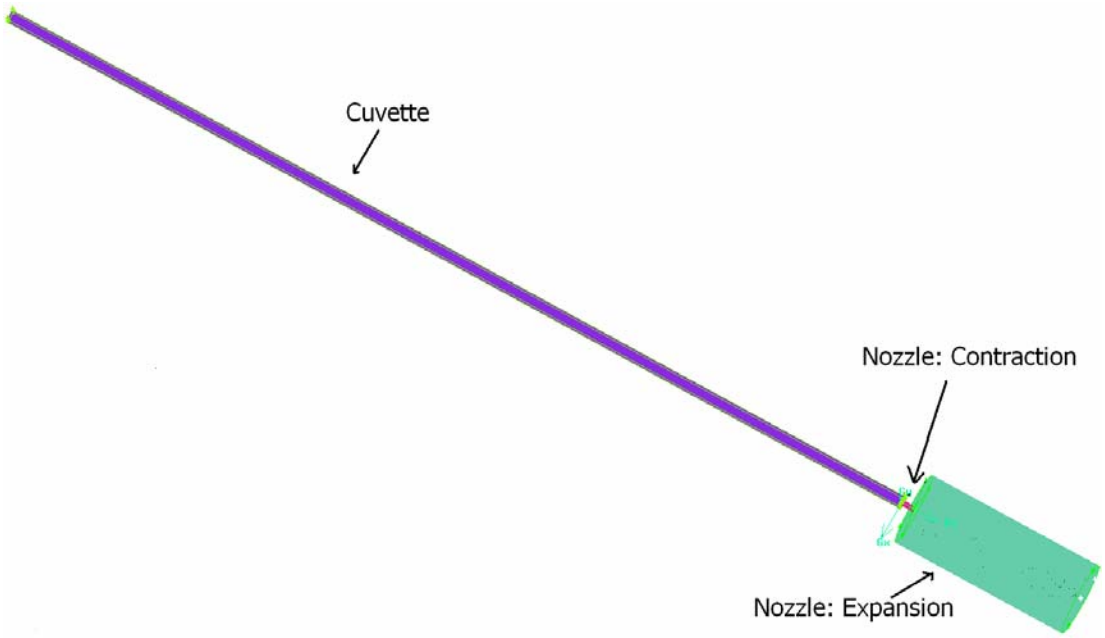


**Figure 23: Mesh of Cuvette and 70 micron Nozzle**

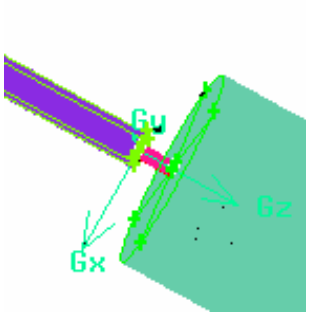


**Figure 24: Connection between the Cuvette and Nozzle**

The completed mesh can then be exported to FLUENT, which simulates the flow through the channel based on the pressure drop as well as a variety of other boundary conditions. This

project established the fluid dynamics of the channel and was able to model the geometry of the FACS Aria; therefore, the next step is to begin simulating the flow through the channel.

## 4. Discussion

### 4.1 Single Passes through Torture Chamber

The results for the single pass experiments in the torture chamber show the sensitivity of the U-937 cell line. Again, this cell line belongs to the same leukemia cell family as THP-1; therefore, it was expected that these cells would perform similarly to THP-1. Figure 8 gives the results of the U-937 cell line alongside the THP-1 results from previous studies, showing that both cell lines behave similarly. Both cell lines seem to have a rapid increase in damage ratios after an EDR of 2.27x10^6 W/m^3; however, U-937 cells appear to be slightly less sensitive than THP-1 as seen at an EDR of 1.09x10^8 W/m^3, where U-937 cells have a 62.3% damage ratio and THP-1 cells have 70.3%. However, this difference is almost negligible when comparing to other cell lines, such as CHO which only undergoes 16.89% damage at an EDR of 1.51x10^8 W/m^3. Since these two cell lines behave similarly, it would be interesting to further investigate this family of cells, the K-562 cell line, in order to conclude that this family is indeed more sensitive than other cell lines. Further studies comparing sensitivity levels of various cell families can also be conducted in order to understand what makes a specific cell line more sensitivity than another.

### 4.2 LDH Analysis of Sorting Experiments

The results for the two sorts with THP-1 using the 85 and 70 micron nozzles display expected trends in the LDH analysis regarding the media difference; however, it is interesting that there seems to be no difference between the two nozzles. When the cells were sorted in media that had 10% FBS, the cells did not undergo as much damage as those cells sorted in 0% FBS. However, the amount of damage that occurs in the 85 micron is approximately the same as the 70 micron, which is interesting because one would think the smaller diameter would apply

more stress to the cells and therefore an increased damage ratio would be observed. It is important to note that there is a large standard deviation, ±11.8% in the average value for the 10% FBS sort with the 70 micron nozzle. The deviation in the other values is significantly lower than this; therefore, it would be interesting to repeat this experiment to try and reduce the amount of deviation. A statistical analysis on the difference between 10% FBS and 0% FBS was conducted using the computer software, JMP. This analysis demonstrated that the 10% FBS sorting media yielded significantly less cell damage than the sorting media without FBS.

4.3 Growth Kinetics after Sorting

Looking at the growth kinetics, one can see a clear difference among the curves. In the first sort, the control cells clearly perform the best and begin growing after the 50 hour mark while the sorted cells in the fresh RPMI with 10% FBS media do not start until after 60 hours. The conditional media cells also do not move into the exponential phase as early on as the control. Clearly, the cells are affected in some manner so as to prevent them from moving into the exponential phase after the sort. However, it is interesting to note that all three samples grow at similar rates once they reach the exponential phase. They move parallel to each other and reach approximately the same concentration in the stationary phase, Figure 12. Additionally, the growth rates that were calculated are similar and show no distinct decrease in the growth rate of the conditional media or fresh media in comparison to the control cells. The fourth sort demonstrates a similar trend for the THP-1 cells in that the growth rates are similar but the control cells move into the exponential growth phase before the sorted cells.

Analyzing the growth kinetics for the U-937 sort is different from the sorts with the THP-1 cell line. The kinetics did not include conditional media because not enough cells were available to seed the flasks. Additionally, the flasks for the sorted cells had some kind of

bacterial contamination that was observed after the 150 hour sampling. As can be seen in Figure 17, the cells died after the contamination occurred; therefore, the kinetics analysis for the sorted cells was stopped after this. However, the important region is the growth until the contamination occurred. Looking at this region, Figure 18, one can see a similar trend as the THP-1 cell lines with the control cells moving out of the lag phase ahead of the sorted cells in the 10% FBS media. Again, it was seen that the growth rates are not affected by the sort, as the growth rate of the control and sorted cells in the fresh media are similar.

The growth kinetics analyses clearly show a difference between the control and sorted cells; however, another significant observation involves the marked difference between the conditional media and the fresh media. Both of these were seeded with sorted cells; however, the conditional media grows exceptionally better than the fresh media. Throughout the growth kinetics the cells in the conditional media remain in between the control and the fresh media cells. This improved performance could be due to the presence of growth factors in the old media from maintaining previous cell growth. Further investigation into this performance could yield a better understanding of what factors may be present in aiding cell growth and a new seeding technique for cells after sorting.

The glucose and lactate concentrations, which complement the growth of the first sort, Figures 13 and 14, portray expected growth characteristics. As the cells feed on the carbohydrate source, glucose, they produce the lactate. In Figures 11 and 12, one can see that the growth levels off into the stationary phase around 160 hours, and the glucose and lactate concentration changes seem to slow down close to this point. Unfortunately, due to problems with the measuring instrument, the YSI Biochemical Analyzer, the glucose and lactate concentrations could not be measured for the entire duration of the first sorting growth kinetics

or for the rest of the sortings. However, some clear trends exist. The conditional media starts with different concentrations of these sugars because it is comprised of 50% old media from which glucose has already been consumed and some lactate produced. Additionally, there does not appear to be any major difference in glucose consumption/lactate production between the sorted cells in the fresh media and the control cells.

4.4 Cell Cycle Analysis

The results of the cell cycle analysis provide interesting observations about the damage that occurs both in how it affects the cells in the G2 phase and potential differences between the two nozzle sizes. The first sort with the 85 micron diameter nozzle did not exhibit the expected results with a decrease in the cell population in the G2 phase: before the sort 19.66% of cells were in the G2 phase and afterwards there still remained 19.29%. However, there is a marked difference between the graphs for before and after the sorting. Before the sorting experiment, the cell cycle graph portrays sharp, distinct peaks as markers for the different phases, but after the sort was performed, the peaks a wider and not as distinct. This shows that the stress has some kind of effect on the cell cycle, but does not necessarily cause a reduction in the G2 phase when cells are sorted with the 85 micron nozzle. When the cells were sorted using the 70 micron nozzle, a marked difference could be seen: before the sort 19.14% of the cells were in the G2 phase and afterwards only 15.6% were present. This result coincides with previous results concerning cell cycle analysis and sorting in the Aria with the 70 micron nozzle. Further trials need to be conducted in order to test whether this result in the reduction of the G2 phase is statistically significant. The lack of reduction in the G2 phase for the 85 micron nozzle shows that there may be a difference in the hydrodynamics of the nozzle with the larger diameter. One possible difference involves the level of EDR that the nozzles may exert on the cells, and future

simulation work would be able to confirm any difference that exists here. When considering the geometry of the channel the slight increase in the diameter could provide a significant decrease in the EDR. A high EDR could be the reason for the reduction in the G2 phase that is seen in the 70 micron sorting.

## 4.5 Modeling

From Figures 23 and 24, one can clearly see the complicated nature of the flow through the FACSAria. Some limited drawings of the cuvette and other components were provided; however, they did not clearly show the transition between the different components nor was did they provide any information about the interior of the nozzle. Therefore, much of the geometry modeling was based on in-house measurements and an understanding how the FACS works. Once a sketch of the geometry was established, modeling in Gambit began; however, the lack of a smooth transition among the components caused problems with meshing the geometries together. Instead of funneling the flow from the cuvette to the nozzle, the rectangular cuvette and circular nozzle are forced together with an o-ring. This could lead to interesting flow characteristics and sharp differences between the 85 micron and 70 micron nozzles due to the fact that a larger nozzle diameter will lead to a smaller immediate contraction. Simulations will be greatly useful for determining the differences between these nozzles; however, due to the difficulty in modeling, the simulation stage of this research has not been reached. The project will continue, and now that meshes have been created, future researchers can perform the actual simulations of the channel with both nozzle diameters: 85 and 70 microns.

## 5. Conclusions and Recommendations

This research focused on the cell damage that occurs during the cell sorting process. Several important conclusions regarding cell sensitivity and response to stress can be drawn from this study. Additionally, some conclusions and recommendations can be made regarding the modeling and future simulation aspect to this research.

From the three single pass experiments, the results were averaged and graphed in order to compare the behavior of the U-937 cell line to THP-1 and other cell lines. The U-937 cell line displays similar characteristics as the THP-1 in terms of response to hydrodynamic stress. This is significant because it shows that cell lines belonging to the same family have similar levels of sensitivity to stress. Further investigation into this family, including an analysis on the K-562 cell line, must be done to evaluate the behavior of this family of leukemia cells. Additionally, future research should investigate cell lines from different families to understand how cells respond to different levels of stress.

The LDH analysis of the cell sorting experiments yields a significant conclusion regarding the effect that media used during sorting has on the amount of damage. The results show that the 10% FBS media performs much better than the 0% FBS media in protecting the cells from damage due to the cell sorting. This study showed no difference between the two nozzle sizes in the amount of damage, based on the LDH analysis, inflicted upon the cells. However, the significant standard deviation in the result for the 70 micron sort with 0% FBS gives probable cause for repeating this experiment. Overall, more sorting experiments must be done with both nozzle sizes to accurately conclude that there is no difference in the amount of damage.

From the growth kinetics analyses, one can clearly conclude that the control cells transition into the exponential growth phase from the lag phase earlier than the sorted cells. This provides a reason for the slowed growth of cells that researches have seen after sorting. The sorting with U-937 cells must be repeated because contamination occurred with the sorted cells, and although the cells appear to follow the general trend with cell growth after sorting, some different results may have been observed due to the contaminant presence. Additionally, it is important to note that the conditional media performs better than the fresh media, meaning that instead of seeding sorted cells in fresh media, researchers could take advantage of presence of growth factors in the old media and seed cells in the 50/50 media after sorting to improve cell growth. Although the results of the growth kinetics for the THP-1 cells provide substantial evidence for the conditional media, more experiments must be made with U-937 and other cell lines in order to conclude that this improved performance is a general feature and not unique to THP-1 cells.

The cell cycle analysis yields an interesting conclusion regarding the difference between the 85 micron and 70 micron nozzles. In all other results, there does not appear to be a significant difference between nozzle sizes; however, here a marked difference between the nozzles is observed in the reduction of the cell population in the G2 phase. The 70 micron nozzle displays expected results and a decrease in the amount of cells in the G2 phase is seen; however, this does not hold true with the 85 micron nozzle. Further investigation into this discrepancy must be made, but from the 70 micron results, one can conclude that the G2 phase is more sensitive to damage due to cell sorting, and therefore hydrodynamic stress.

Finally, the modeling conducted in Gambit established the necessary geometries and fluid dynamics of the channel in the Aria. Therefore, simulations must be done with both meshes (85

and 70 micron nozzles) to establish the level of EDR in the Aria. From this, the amount of EDR in the Aria can be related to the amount of cell damage that was observed in this study. Additionally, differences in the amount of EDR inflicted with both nozzles must be investigated because of the interesting results seen in the LDH and cell cycle analyses in that there did not appear to be a significant difference between the sizes according to the LDH analysis but a distinct disparity was observed in the cell cycle analysis.

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